THE YALE JOURNAL OF BIOLOGY AND MEDICINE 57 (1984), 865-868

Cloning by Limiting Dilution: An Improved Estimate That an Interesting Culture Is Monoclonal

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Received March 16, 1984

An interesting culture obtained by limiting dilution is less likely to be monoclonal than a random viable culture.

Current practice using limiting dilution to establish monoclonal lines of interesting recombinant DNA or hybridoma-derived organisms overestimates the probability that promising cultures are monoclonal, resulting in inadequate dilutions, with the need for additional subcloning and the avoidable loss (avoidable instability) of interesting lines by overgrowth with uninteresting varieties.

In cloning by limiting dilution [1] it is often assumed that progenitors are Poisson-distributed and the probability that a resulting interesting culture is monoclonal is the same as that of any viable culture [2]. An improved estimate of the probability that a culture containing interesting cells is monoclonal can be obtained independent of cloning efficiency by assuming interesting and uninteresting progenitors independently Poisson-distributed and calculating the conditional probability that an interesting culture arose from a single progenitor. The probability an interesting culture is monoclonal is calculated in terms of the fractions of sterile and uninteresting cultures and shown to be bounded above by that of a random viable culture, decreasing with increasing rarity of interesting cultures, and bounded below by the fraction of sterile cultures. The improved estimate is likely to be useful in the large-scale cloning to create monoclonal lines which is routinely done in recombinant DNA and hybridoma work.

Assuming progenitors independently Poisson-distributed with average number x interesting (the definition of interesting is left arbitrary) and y uninteresting per culture, the probability of having placed i interesting in culture with j uninteresting is:

$$P(i,j) = P_x(i)P_y(j) = e^{-x}\frac{x^i}{i!}e^{-y}\frac{y^j}{j!} \quad [1]$$

The probability a culture containing interesting cells is monoclonal is:

$$P_M = \frac{P(1,0)}{1 - P_x(0)} \quad [2]$$

This paper received honorable mention in The Yale Journal of Biology and Medicine Student Research Paper Award, May 1984.

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Expressed in terms of the fractions of sterile $s = e^{-x-y}$, and uninteresting (including sterile) $u = e^{-x}$ cultures, the equation becomes:

$$s \leq P_M = \frac{s \ln u}{u - 1} \leq \frac{s \ln s}{s - 1}, \quad 0 \leq s \leq u \leq 1 \qquad [3]$$

(since $\frac{\ln u}{u - 1}$ is a decreasing function of u with $\text{limit}_{u \to 1} = 1$).

The upper bound $\frac{s \ln s}{s - 1}$ represents the case where interesting is defined as viable ($u = s$) and is the widely used optimistic estimate of P_M referred to above [2].

Since the cultures are independently distributed, cloning by limiting dilution is equivalent to the imaginary process of first aliquoting the interesting and then the uninteresting progenitors. After the first step, uninteresting cultures are sterile and the probability an interesting culture is monoclonal is [1] $\frac{u \ln u}{u - 1}$. After the second step, $\frac{s}{u}$ of the initially sterile (and nonsterile) cultures did not receive uninteresting progenitors, so $P_M = \frac{s}{u} \frac{u \ln u}{u - 1}$.

The lower bound is easily understood by considering the case where interesting progenitors are so rare they do not appreciably affect the fraction of sterile cultures ($u = 1$). An interesting monoclonal culture can only arise from a single progenitor placed in sterile culture, so P_M here is simply s.

The probability that an interesting culture is monoclonal is less than or equal to that of a random viable culture, decreases for increasingly rare interesting cultures, and is bounded below by the fraction of sterile cultures.

This result is significant since the common intuitive notion (that the probability an interesting culture is monoclonal is the same as that of a viable culture) is shown to be a gross overestimate in many circumstances (refer to Table 1).

In subcloning so that interesting subcultures have a probability of being mono-

TABLE 1

Probability a Culture Containing Interesting Cells is Monoclonal

$$P_M = \frac{s \ln u}{u - 1}$$

					s					
	0.1	.2	.3	.4	.5	.6	.7	.8	.9	1.0
0.1	26%									
.2	20	40								
.3	17	34	52							
.4	15	31	46	61						
.5	14	28	42	55	69					
u .6	13	26	38	51	64	77				
.7	12	24	36	48	59	71	83			
.8	11	22	33	45	56	67	78	89		
.9	11	21	32	42	53	63	74	84	95	
1.0	10	20	30	40	50	60	70	80	90	100

P_M, the probability an interesting culture obtained by limiting dilution is monoclonal (rounded to the nearest percentage) is expressed in terms of the fractions of sterile s and uninteresting (including sterile) cultures u. The first entry in each column ($u = s$) is the widely used estimate of P_M referred to above [2].

clonal of at least P_C they should be aliquoted with an average of N progenitors per subculture to produce a fraction sterile $S = e^{-N}$ such that:

$$P_C \leq 1 - (1 - P_M)(1 - S) \quad [4]$$

Expressed in terms of N:

$$N \leq \ln \frac{1 - P_M}{P_C - P_M} \quad [5]$$

To ensure a probability P_V of at least one viable subculture, at least V subcultures must be aliquoted such that

$$P_V = 1 - e^{-NV} \quad [6]$$

Solving for V:

$$V = \frac{-1}{N} \ln (1 - P_V) \quad [7]$$

In any application, s and u must be approximated. Fractions $\bar{s} = S/T$ and $\bar{u} = U/T$, where S and U are the binomial distributed numbers of sterile and uninteresting (including sterile), and T the total number of cultures, respectively, are sample random variable averages (approximately normally distributed for large T) with means s and u and variances $s(1 - s)/T \leq 0.25/T$, and $u(1 - u)/T \leq 0.25/T$, respectively. Choosing $s = \bar{s} - T^{-1/2}$, $u = \bar{u} + T^{-1/2}$ ($T^{-1/2} \geq$ two standard deviations) will provide a conservative working estimate of P_M and N.

Current practice using limiting dilution to establish monoclonal lines of interesting organisms derived through recombinant DNA or hybridoma technology overestimates the probability that promising cultures are monoclonal, resulting in inadequate subcloning dilutions with the need for additional subcloning (wasting time, effort, and materials) and the avoidable loss of interesting lines by overgrowth with uninteresting contaminants (explaining some of the notorious instability of newly established "monoclonals").

For example, suppose in a large hybridoma experiment fusomas are aliquoted to 10,000 culture wells with 7,000 wells showing growth at two weeks, supernatants of 2,000 of those assaying as antigen-specific (interesting) by ELISA.

In this case $T = 10{,}000$, s and u may be directly approximated with $P_M = 0.33$ by equation [3] above or Table 1 with $s = 0.3$ and $u = 0.8$. Current practice optimistically estimates $P_M = 0.52$, assuming $s = 0.3$ and that the probability an interesting culture is monoclonal is the same as any other viable culture, $s = u = 0.3$ in equation [3] above or Table 1.

In subcloning to ensure the probability that an interesting subculture has at least 95 percent probability of being monoclonal, the subcultures should be aliquoted with less than an average of $N = 0.078$ progenitors per subculture and at least 59 subcultures aliquoted to ensure a 99 percent probability of at least one being viable by equation [5] and equation [7] above or Table 2.

Under these conditions the logic of current practice dictates aliquoting subcultures with an average of less than N progenitors per subculture such that:

$$0.95 \leq 1 - (1 - 0.52)\left(1 - \frac{S \ln S}{S - 1}\right) \quad [8]$$

TABLE 2
Maximum Average Number of Progenitors Aliquoted per Subculture to Ensure 95 Percent Probability Interesting Subcultures Are Monoclonal

$$N = \ln \frac{1 - P_M}{P_C - P_M},\ V = \frac{-1}{N} \ln (1 - P_V),\ P_C = 0.95,\ P_V = 0.99$$

					s				
	0.1	.2	.3	.4	.5	.6	.7	.8	.9
0.1	$.069_{67}$								
.2	064_{72}	087_{53}							
.3	062_{74}	079_{59}	109_{43}						
.4	060_{76}	074_{62}	096_{48}	137_{34}					
.5	059_{78}	071_{65}	089_{52}	119_{39}	177_{26}				
.6	059_{79}	069_{67}	084_{55}	107_{43}	148_{31}	240_{20}			
u .7	058_{79}	067_{68}	080_{57}	100_{46}	131_{35}	191_{25}	353_{14}		
.8	057_{80}	066_{70}	078_{59}	094_{49}	120_{39}	163_{29}	259_{18}	626_{8}	
.9	057_{81}	065_{71}	075_{61}	090_{51}	111_{42}	146_{32}	211_{22}	383_{13}	3.383_{2}
1.0	057_{81}	064_{72}	074_{63}	087_{53}	105_{44}	133_{35}	182_{26}	287_{17}	693_{7}

Each entry is of the form $N_{,v}$ where N is the average number of progenitors per subculture (rounded down to three decimal places) so that the probability an interesting subculture is monoclonal is at least $P_C = 0.95$, and V is the number of subculture volumes (rounded up to integers) aliquoted to ensure a probability $P_V = 0.99$ at least one will be viable. $P_M = \frac{s \ln u}{u - 1}$, is the probability the original interesting culture is monoclonal, where s and u are the fractions sterile and uninteresting (including sterile) of the original cultures, respectively.

which may be solved by iteration with $S = 0.807$ and $N = 0.214$. If aliquoted at this concentration, the probability that an interesting subculture is monoclonal may be calculated by equation [4] above as

$$P_C \leq 1 - (1 - 0.33)(1 - 0.807) = 0.87$$

At $N = 0.078$, 5 percent of interesting subcultures are at risk of overgrowth with contaminants, whereas at $N = 0.214$, at least 13 percent are potentially unstable, representing as much as a 2.6-fold loss over that anticipated.

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